**TNO Quality of Life**

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**Subject**
Expert Panel Report ICCVAM/NICEATM

**Date**
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**Our reference**
TAP-2005

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**Attachments**
Annex (8 pages)
Pathologist report (1 page)
Intralaboratory report (17 pages)

Dear Sir,

In reaction to the request for comments made public via the Federal Register notice, Volume 70, No. 53, Monday, March 21, 2005/Notices, 13513, the Dutch Research Organization TNO would like to forward the following comments and remarks concerning the ICE test method described in the Expert Panel Report: "Evaluation of the Current Validation Status of In Vitro Test Methods for Identifying Ocular Corrosives and Severe Irritants".

TNO has a long-standing position in fundamental toxicological research in Europe and is recognized by industry and government as a reliable and independent contract research organization (CRO) for full regulatory and testing services. TNO supports registration of chemicals and pharmaceuticals in compliance with the relevant guidelines issued by various international authorities, like the OECD, EU, EPA and FDA. For that purpose, the Toxicology and Applied Pharmacology Department of TNO employs the ICE test system, described in the Panel Expert Report, as a pre-screen for all levels of eye irritancy for their standard contract OECD 405 eye irritation testing in rabbits. From the early 90's, the European regulatory agencies accept *in vitro* screening of severe eye irritants by using isolated eyes or corneas or the Hen's-egg chorioallantoic membrane (HET-CAM assay). Therefore, the ICE test method is officially replacing the Draize Eye Test in case of severe eye irritancy for all compounds tested at TNO since 1992.

It is therefore, that TNO would like to respond officially and extensively to the request for comments. As the main user of this test system TNO has a long practical history in the detection of severe eye irritants. As such TNO highly appreciates the initiative of ICCVAM/NICEATM to evaluate four of the most currently used alternative test methods (HET-CAM, BCOP, ICE and IRE). The expert report represents the results of extensive and comprehensive evaluation of these methods performed by the ICCVAM members and a panel of experts and contains valuable information needed for a successful introduction of these methods.
At the same time, we have to conclude that TNO's previous input with respect to the ins and outs of the test method has not always been taken seriously into consideration. This conclusion is based on the nature of the assessments, recommendations and remarks made in the document and the way the ICE test method was discussed during the ICCVAM Ocular Expert Panel meeting, January 11-12, 2005 in Bethesda, USA.

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TNO has been involved in the international validation process of alternative test systems for eye irritation since the very first introduction of these methods in the early eighties. Throughout that process, the participants learned the extreme importance of exchanging information between experts, especially concerning the practical aspects of the test methods. In that light, it is regrettable that the offer of TNO to organize and host a meeting and demonstration of the ICE test method for key experts of the panel prior to the expert meeting was declined by ICCVAM. We feel that the presence of the panel experts would have been invaluable for understanding the practical aspects of the test method and would probably have avoided considerable discussion about certain practical aspects of the method.
To give an example, the panel recommends to use centering lights on the optical pachymeter to mark and fix the center reading of corneal thickness to improve these measurements across laboratories. If the panel would have experienced the ICE test method, it would immediately have been clear that the center of the cornea can be located and measured without any problem whatsoever. The reason for the variation in measurement across laboratories signaled by the panel is of a quite different nature and is further discussed in our comments. Inclusion of centering lights would be a measure, not only superfluous, but also expensive, time-consuming and unnecessarily complicating the travel of the method between laboratories, especially giving the fact that the method is intended to act in the highest spectrum of irritancy. More examples of these kinds of misunderstanding or misinterpretation exist and are discussed in the comments. We would also like to point out that throughout the expert panel report, identical subjects, such as the "accuracy and reliability of the *in vivo* rabbit eye test", are described and handled quite differently for the 4 different *in vitro* test systems. The report could benefit from more consistency in that respect.

Early February 2005, with the highly appreciated support of ICCVAM, TNO retrieved from their archives the individual parallel *in vivo* Draize Eye Test and *in vitro* ICE data of 94 compounds tested simultaneously within the scope of screening out severe eye irritants for EU legislation. These data were submitted and TNO is confident that the evaluation and inclusion of the data will lead to certain updates in the report. Furthermore, data concerning the intra-laboratory repeatability of the ICE, assessed during the Reference Standard Validation of *in vitro* tests sponsored by ECVAM, were forwarded by ECVAM to ICCVAM in February 2005. This data set should also be taken into consideration for updating the paragraph of the report.

Specific comments, ordered by section or paragraph, are mentioned in the attachment to this letter. TNO is confident that they will help to further improve the quality of the expert report and the introduction of the ICE and the other three methods as a standard screen for severe eye irritancy.

We are looking forward ICCVAM's official reaction to our comments with great expectation. In the mean time, TNO is, as always, available for additional information and discussion, if needed.

Yours faithfully,

Dr Ruud A. Woutersen
Head of the Business Unit Toxicology and Applied Pharmacology, TNO Quality of Life

1.1.1 2nd line. The ICE is not intended as a screening assay for severe irritants only. It is used as a screen for the full range of irritancy, but is allowed as a screen for severe irritants in Europe.

2nd paragraph. In contrast to what is mentioned, the mechanistic basis for eye irritation/corrosion is known, i.e. as mentioned in section 1.1.2 "chemical interaction of the cornea with the test compound causing coagulation/erosion of epithelial cells, stromal oedema as a result of loss of the barrier function of the epithelium, disruption/alteration of stromal fibers causing opacity, in severe cases damage to the endothelial layer also causing stromal oedema". These mechanisms are identical, irrespective of the origin of the cornea, i.e. human, rabbit, bovine, pig or chicken corneas. The expression in corneal swelling, opacity and fluorescein retention/penetration in the different species largely depends on differences in the general structure of the cornea, i.e. for instance the number of epithelial cell layers which can be quite different especially in the bovine compared to other species. Hence, the difference in contact time between the ICE and BCOP – 10 seconds versus 10 minutes – needed to induce the same irritating response. This paragraph should be rewritten.

1.1.2 page 30, 2nd line. The Draize test has never had any data provision or scoring system for the anterior chamber of the eye!

page 30, line 6-8. What is the rationale of this remark. The *in vivo* test itself does not allow for the definition of the mechanisms of corneal opacification. Moreover, the ICE or BCOP offers more possibilities for this assessment by using histopathology by which coagulation or oedema or the combination as the cause for opacity can be determined.

2nd paragraph. There is a general misconception that the isolated eye test measurements covers the actual situation *in vivo*. It is obvious that the ICE, IRE or BCOP cannot cover processes *in vivo* that evolves over days after exposure, it is not intended to do so. The isolated eye test measures certain parameters which come to full expression over a four-hour observation period. Longer observations are useless and without meaning, on the one hand because there is a limitation to the storage life of the eyes under these conditions and on the other because after this period there is no additional relevant expression of effects. The claim that damage to the endothelium is likely to take longer is speculative and should be substantiated or removed from this paragraph. It is as likely to assume that, if the test chemical can cause substantial damage to the epithelium and stroma in a few seconds, it can do the same to the endothelium. Stromal oedema is the resultant of a passive process (epithelial damage) and/or an active process (disturbance of the active pump function of the endothelium) and as such measured as corneal swelling in the ICE/IRE. Topically applied compounds only damaging the corneal

endothelium without damaging the other layers of the cornea are according to our knowledge not known.

3rd paragraph. It is obvious that the isolated eye test systems cannot screen conjunctival effects, although Burton, the inventor if the isolate eye test, already pointed out in his publication of 1972 that corneal thickness correlates well with conjunctival effects (based on *in vivo* assessments with 100 compounds in 600 rabbits!). This correlation is also confirmed in the 1996 publication of Prinsen (based on the parallel *in vivo/in vitro* ICE testing). There is no relevance in stating that the 4-hour ICE observation is too short to screen conjunctival effects by mediators, because the ICE does not claim this kind of screening at all. The remark that the ICE/IRE/BCOP does not directly measure conjunctival effects is sufficient and it would do more justice to the ICE/IRE assay to quote the publication of Burton on this issue. For that matter, it would also be better to discuss the relevance of the conjunctival effects *in vivo*. How realistic and relevant is it for human ocular exposure and hazard/risk evaluation to instill 100 ul or 100 mg of a test compound in one's lower conjunctival cul-de-sac.

1.1.3 The presumed (mostly) higher sensitivity of the rabbit eye compared to humans cannot be related entirely to the anatomy or physiology of the eye. The huge differences that exist between exposure conditions in the rabbit Draize Eye Test and in possible exposure in humans should be mentioned and taken into consideration.

Page 31, 2nd paragraph: see remarks 1.1.2.

2.1.1 1st paragraph. The measurement of morphological effects such as loosening/detachment of the epithelial layer is an important parameter for assessment of severe irritancy (also mentioned in the scoring system) and should be mentioned.

2nd paragraph. Baseline opacity measurement (i.e. absence of opacity) is one of the most important predose measurement for inclusion of the eye in testing and must be mentioned in this section.

3rd paragraph. Rejection rate (usually 8%, occasionally up to 45%) and six to twelve heads are necessary to obtain 11 or 12 usable eyes (if a 12-chamber superfusion apparatus is used).

4th paragraph. Other bathing solutions, see remark at 2.1.3.

Page 33. Three eyes to be used for a negative control is a non-issue, especially in the case of severe irritancy. In other test systems (like the BCOP) negative controls are necessary to provide baseline measurements. They are used in the

BCOP because these values are needed as a baseline value for subtraction from the values of the test eyes and because corneal observation as done by slit-lamp microscope is not possible. In the ICE, each test eye provides its own baseline control value. One control eye for monitoring general conditions during the test is always included. During the hundreds of experiments carried out there were never any unusual findings. One control eye per test run is therefore adequate. Furthermore, with 11 chambers there is a possibility of including a second control. The individual data of the 94 compounds sent recently, can be reviewed for control eye data. The control data shows that it is not a point of concern. If hundreds of independent test runs with one control eye showing no opacity, no swelling and no fluorescein retention (EC/HO study, in-house validation and test data, etc), what kind of improvement is believed to be obtained from including three control eyes. The panel should make this clear, otherwise it will be a waste of time and money.

Page 33. Bullet no. 1. The temperature is well-controlled, the saline drip is easily adjustable and the chicken cornea is superfused entirely. How did the panel came to this conclusion without having any practical experience with this isolated eye system.

Bullet no. 2. Originally Burton used 4 test eyes. The ICE started with 5 test eyes and after using this protocol for several years it was decided to go back to three eyes, which still resulted in sufficiently reliable data. The set of 94 compounds having 5 and 3 test eye data should prove this. Moreover, other test systems like the BCOP, HET-CAM, the *in vivo* rabbit eye and skin irritation all use 3 replicates (what is sauce for the goose .....).

Bullet no. 3. Again this suggestion is not based on any expertise with the test method. The exposure to the test substance for 10 seconds is done with the cornea in a horizontal position outside the chamber. Only after the rinsing procedure with 20 ml of saline has been completed, the eye is returned to the chamber. Removing and returning the test eye from the chamber has no influence on the 10-second application whatsoever. In its present position, the cornea is adequately superfused during the entire 4-hour experimental period and the 10-second application can be performed without any problem. Without proof of the opposite, this part should be removed. Moreover, it would be better to consider the fact that it is of more importance to keep the cornea in its physiological plane during the 4-6 hour test period.

2.1.3 Fluorescein retention vs penetration. The penetration of fluorescein is not scored at t = 30 min, because this would imply scoring how far (in which layer of the cornea) the fluorescein penetrated within a defined period of time. This would require scoring of fluorescein staining over multiple time points As can be read from the scoring system the amount of fluorescein retained by the damaged epithelium at a specific time point (t = 30 min) is measured,

hence "fluorescein retention" and not penetration. If really being semantic, the term fluorescein "staining" could be used.

HBBS vs isotonic saline. The remark about the use of isotonic saline drip and possible increased permeability or even sloughing off the corneal surface is purely speculative. The validity of isolated rabbit eyes kept superfused in isotonic saline was already adequately examined and confirmed by Burton in its publication of 1981. Since then over hundreds of experiments with isolated rabbit and chicken eyes have never shown any increased permeability, let alone sloughing off the corneal surface. Investigators of the isolated eye test have never reported such events to occur with saline superfused corneas. These kind of speculations should be removed from the report or be substantiated. Recently, a test compound in the lower range of irritancy was examined by 3 test eyes and 1 control eye superfused with saline and 3 test eyes and 1 control eye superfused with HBBS without any significant difference in result (raw data included in the deliverance of the letter and this annex by mail courier).

| Superfusion | Maximum mean score for: | | | Irritation categories[1] | Irritation Index[2] | Irritation Classification |
|---|---|---|---|---|---|---|
| | Swelling % | Opacity | Fluorescein retention | | | |
| physiological saline | 4 | 0.8 | 1.0 | I;II;II | 40 | Slightly irritating |
| HBSS | 5 | 0.8 | 1.0 | I;II;II | 41 | Slightly irritating |

1 I = no effect; II = slight effect

2 Irritation Index = maximum mean corneal swelling + maximum mean opacity (x 20) + mean fluorescein score (x 20)

Fluorescein measurement. The use of the microscope guarantees a very accurate measurement of the retention/stain of fluorescein (even individual single cell staining can be discriminated). Moreover, the qualitative assessment of the retention/staining is as (or more) important as the quantitative assessment and cannot be measured by sensor system.

2.1.4. Again this section outlines a false picture of the actual situation. There is no prior manipulation influencing the exposure time. The 10-second application is and can be performed accurately.

2.1.5. Solids that adhere to the cornea *in vivo* also adheres to the cornea *in vitro*; this is not the problem. The problem is that *in vivo* the solids are entrapped in the

conjunctival cul-de-sac and can stay there for up to 24 hours. This is a general problem extremely hampering the validation of *in vitro* methods. By now, it should be realized that *in vivo* this is an unrealistic, unscientific (and unethical) situation. For hydrophobic compounds it is the opposite, i.e. mostly a very short exposure *in vivo* and a more pronounced effect *in vitro* probably, because of a lack of the animals' defense mechanism (including the Tear film). Mostly these hydrophobic compounds show distinct skin irritation because of the skin exposure under (semi)occluded conditions (Prinsen, in-house parallel eye *in vivo/in vitro* and *in vivo* skin irritation).

2.1.6. Nature of the response assessed. The last sentence makes no sense and should be removed or rewritten. The parallel data presented in the 1996 publication of Prinsen and the additional data set recently sent confirm its predictive capacity, especially for the non-irritants. Moreover, these remarks are not mentioned in the same section of the BCOP and not at all discussed in the HET-CAM part of the report, whereas the "Nature of the response assessed" is identically relevant for all 4 *in vitro* methods.

2.1.7. Negative controls; see remarks at 2.1.1. The last sentence makes no sense since control eyes are not dosed, but only rinsed.

2.1.8. Solvent control (also mentioned in 2.1.7). Solvents are never used because the substances are tested neat (the document even mention this in section 2.1.2).

2.1.12 The irritation index is neither the primary tool for statistical evaluation nor is it used for classification of the eye irritancy. It is only used for ranking purposes. It was invented under pressure of the EC/HO management team because validation of the *in vitro* assays was initially to be performed with one single index only (*in vivo* MMAS and an *in vitro* index). Because all three parameters weigh equally in the official classification scheme of the ICE, the score for opacity and for fluorescein must be multiplied with a factor 20.

2.1.13 It is unclear why ICCVAM concludes that there is no real tissue change parallel between the ICE test and *in vivo* rabbit eye test data. The Prinsen (1996) publication, which is available to ICCVAM clearly demonstrates the parallel by comparing the individual tissue scores of the *in vivo* Draize test and the *in vitro* ICE. Why are these data not taken into account. Moreover, ICCVAM received data of another 50 compounds with parallel data which further substantiate the existing link between corneal damage *in vitro* and *in vivo* corneal and conjunctival effects (see also final comment 1.1.2).

2.4 The individual ICE data from the EC/HO data (n=5) and the parallel *in vivo*/ICE data (n =5 and n=3) are made available to ICCVAM. Therefore, the decision for the reduction made by Prinsen can be verified. Moreover, the current OECD practice allows classification of a severe irritant, already on the

basis of a single animal. Because, in the scope of ICCVAM, the ICE will only act in the high end of the irritancy spectrum, it seems unjustified to insist on five test eyes (the 3 test eye system has even a longer history than the five eyes). Furthermore, ICCVAM should consider the fact that, originally, the method was developed by Burton (1980) to be carried out with 4 test eyes. Also the other current candidates use 3 replicates for their assessments, while the source of tissue is as "unpredictable" as that of the ICE (chicken eyes or bovine eyes).

The last paragraph is speculative and not founded by ICCVAM and should be removed. Eyes that are selected for testing must meet absolute high standards, i.e. absolutely free from corneal opacity, free of fluorescein staining and thickness within the normal range and no swelling during the acclimatization period.

2.6 See earlier comments.

2.7 1st bullet. The correction factor for swelling was introduced at the time of the EC/HO study, because it appeared that different slit-lamps used by the participants, can produce different thickness values when not controlled or standardized for the slit-width. Furthermore, the measurement is made by the exact positioning of the lower slit against the upper slit, which can be a cause of variation. In the area of severe irritancy these factors will not be of that much concern. Normal practice for any newcomer would be to test its test system with a reference set of chemicals covering the full range of irritancy, determine its overall swelling range and subsequently define the threshold swelling values belonging to none, slight, moderate and severe irritation.

2nd bullet. A 12-chamber superfusion apparatus can be built easily. Also the harvesting of 12 eyes is considered to be of no problem.
Currently (i.e. already since 1992) 11 and not 10 eyes are sampled. Dissection of an eye and placing it in the chamber takes usually one minute.

4th bullet. See comment 2.1.3

4.3 Original *in vivo* data of 94 compounds (GLP compliant) have been provided meanwhile.

4.6 Page 41, 1st paragraph, last sentence. The remark that the variability of the *in vivo* scores may be considered insignificant for the purposes of this evaluation since it is focused only on the detection of severe irritants, is quite appropriate. However, ICCVAM should also apply this remark to the *in vitro* tests. The proposals or recommendations for changes of the standard ICE made in the report thus far (other medium than physiological saline, 3 vs 5 test

eyes, horizontal position of eyes, etc.) are all of insignificant influence when dealing with severe eye irritants.
In general section 4.6 is too vague with respect to the possible implications of the variability of the Draize eye test in relation to the evaluation of the *in vitro* screens. The whole issue of testing solids *in vivo* is not discussed (in practice almost one-third to half of the test compounds). The unpredictable exposure conditions with solids in rabbits create insurmountable problems with respect to the expected performance of the *in vitro* assays in the range of moderate/severe irritancy. Furthermore, this section dealing with the accuracy and reliability of the *in vivo* rabbit eye test is repeated for each test method but its wording contains considerable differences between the methods. Are there different *in vivo* rabbit eye irritation tests? Apart from referring to the specific *in vitro* method, the content of this section should be similar for all 4 methods. For instance, the remark for the BCOP that there should be more consideration of the variability of the Draize test upon evaluation of the BCOP assay, applies in the same way to the HET-CAM, the IRE and the ICE!

5.2 Individual *in vivo* Draize and ICE data have been provided recently.

5.3 see 5.2

6.1 1st paragraph. The original data are now available to ICCVAM and should be considered.

2nd paragraph. This is the type of conclusion that is highly debatable if the nature of the compound and its *in vivo* exposure condition are not taken into account, as is expressed in our comments on section 4.6.

7.0 see comment 6.1, 2nd paragraph.

7.2 1st paragraph. Data and comments with respect to intralaboratory reproducibility of the ICE which were gathered in the ECVAM reference standards validation program have already been forwarded and should be included in the ICE BRD (again included in the deliverance of the letter and this annex by mail courier).

8.2+8.3+8.4 The individual data have now been submitted to ICCVAM. All studies have been performed under GLP and the Quality Assurance Unit of TNO can provide a statement of GLP compliance, if needed.

11.1 page 49. Vacuum pumps are not required for the ICE test system.

12.1.1 2nd bullet. Data have been submitted now.

6th bullet. See earlier comments on the testing of solids.

12.2.1 1st paragraph. This kind of evaluation can now be performed on the individual data provided recently.

2nd paragraph. The exposure to the test substance for 10 seconds is done with the cornea in a horizontal position outside the chamber. Only after the rinsing procedure with 20 ml of saline has been completed, the eye is returned to the chamber. Removing and returning the test eye from the chamber has nothing to do with the 10-second application whatsoever. In its present position, the cornea is adequately superfused during the entire 4-hour experimental period and the 10-second application can be performed without any problem.

Centering lights. Placing the slit onto the center of the fixed cornea is one of the easiest performances in the isolated eye test. It is unclear on the basis of what kind of experience with isolated eye testing this recommendation has been made. If mentioned, it should be optional.

12.2.2 Currently there is a scoring system for histopathology which is used routinely when the ICE is used as a stand-alone test. An example of the microscopical evaluation of untreated chicken corneas sampled 1, 1½ and 2 hours after kill performed by TNO's board-certified toxicologic pathologist Dr M. Wijnands, is attached.

12.3.2 Minority opinion, item 5. The opinion that not all 4 methods need to be developed further is a considerable underestimation of what these methods represent in the already approved EU screening of severe eye irritants. Moreover, for introduction of alternatives for eye irritancy internationally, there should be a reasonable choice from different test systems since these systems depend on various sources of test material which will not be available equally throughout the world.

# Appendix - Individual microscopical findings

| Sample after kill | Eye number | Epithelium | | Stroma | | | Endothelium |
|---|---|---|---|---|---|---|---|
| | | | | | Pyknotic nuclei | | |
| | | Erosion | Necrosis | Disorder of fibers | outer region (adjacent to epithelium) | inner region (adjacent to endothelium) | Necrosis |
| 1 hr | 1 | - | - | - | - | - | - |
| 1 hr | 2 | - | - | - | - | - | - |
| 1 hr | 3 | - | - | - | - | - | - |
| 1½ hr | 1 | - | - | - | - | - | - |
| 1½ hr | 2 | - | - | - | - | - | - |
| 1½ hr | 3 | - | - | - | - | - | - |
| 2 hr | 1 | - | - | - | - | - | - |
| 2 hr | 2 | - | - | - | - | - | - |
| 2 hr | 3 | - | - | - | - | - | - |

- = not observed; p = present; ½ = very slight; 1 = slight; 2 = moderate; 3 = severe; + = few; ++ = several

Approximately 7 weeks old, male or female chickens (ROSS, spring chickens), body weight range approximately 2.5 - 3.0 kg, were used as eye-donors. Heads of these animals were obtained from poultry slaughterhouse v.d. Bor, Amersfoortseweg 118, Nijkerkerveen, the Netherlands. Heads of the animals were cut off immediately after sedation of the animals by electric shock and incision of the neck for bleeding, and before they reached the next station on the process line. The heads were placed in small plastic boxes (3 heads per box) on a bedding of paper tissues moistened with isotonic saline. Next, they were transported to the testing facility. During transportation, the heads were kept at ambient temperature. Eyes were sampled 1, 1½ or 2 hours after kill and subsequently preserved in a neutral aqueous phosphate-buffered 4 per cent solution of formaldehyde. The corneas were embedded in paraffin wax, sectioned at 5 µm and stained with PAS for histopathological examination.

The three basic structures, i.e. epithelium, stroma and endothelium were examined microscopically. In addition, the integrity of Bowman's and Desmett's membrane (not mentioned in table) was examined.
None of the corneas eyes sampled at 1, 1½ or 2 hours after kill, showed any abnormalities.

Authentication
I, the undersigned, hereby declare that the pathology data presented in this table were compiled by me, and accurately reflect the data obtained.

Dr. M. Wijnands
(Pathologist)

2 May 2005
Date